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Association of −308 TNF-α promoter polymorphism with clinical aggressiveness in patients with head and neck squamous cell carcinoma

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SUMMARY

Genetic polymorphisms in the promoter region of the tumour necrosis factor-α (*TNF-α*) gene are involved in the regulation of the expression levels of its cytokine. Besides, these polymorphisms have been associated with the clinical behaviour of cancer. We investigated the −308 promoter region polymorphisms of the *TNF-α* gene and its association with the clinicopathological factors of a head and neck squamous cell carcinoma (HNSCC) sample. Furthermore, we analysed the impact of all the variables on the overall survival of patients. A sample of HNSCC ($n = 89$) was evaluated. Clinicopathological factors and overall survival data were gathered. The *TNF-α* gene was analysed by using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP). Data analyses were performed by using bivariate and multivariate statistical tests. Significance was set at $p < 0.05$. HNSCC subjects carrying the A allele (GA/AA) exhibited associations with poor performance status (OR = 2.82, $p = 0.039$), lesions located on posterior areas (OR = 4.02, $p = 0.002$), and large-size tumours (OR = 2.91, $p = 0.015$). Subjects carrying only AA genotype exhibited association with poor performance status (OR = 6.667, $p = 0.007$). A worse overall survival was noted in subjects with large tumours (OR = 4.87, $p = 0.005$) and locoregional metastatic disease (OR = 2.50, $p = 0.018$). Our data suggests that the presence of the A allele/AA haplotype in HNSCC individuals might contribute to the higher clinical aggressiveness of malignant disease.

Introduction

Head and neck cancer represents a broad term that encompasses a group of human malignancies that arise in the epithelial lining of the upper aerodigestive tract mucosa. It represents the sixth most common type of human cancer, and it is responsible for high death rates worldwide every year.[1–7] About 90% of the head and neck cancers are diagnosed as squamous cell carcinoma (SCC).[8] A plethora of socio-demographical, economic, and cultural factors associated with a background of genetic disturbances are pivotal for the incidence and development of head and neck SCC (HNSCC).[8–10]

The rate of tumour development is regulated by a delicate balance between pro- and anti-tumourigenic activities promoted by the neoplastic cells themselves as well as other cells located in the surrounding microenvironment. From this cellular variety are produced several cytokines that modulate the local/systemic immune response that inhibits or stimulates the development of cancer.[11,12] The human tumour necrosis factor-alpha (*TNF-α*) gene is located in the chromosome six region (p21.231), within the class III region of the major histocompatibility complex.[13,14] TNF-α has multiple well-recognised biological activities in many physiological processes such as cell differentiation, proliferation, apoptosis, energy metabolism, and immune status.[13,15] From a pathological point of view, TNF-α has been noted as an important regulator of some inflammatory diseases and malignancies.[13,14,16] In cancer studies, it has been observed that TNF-α might act in paradoxical roles. Therapeutically, a high local dose of TNF-α seems to have powerful antineoplastic actions.[17] However, when chronically produced, this cytokine seems to act as an endogenous regulator that promotes the tissue remodelling for tumour growth and spread.[17–20]

The *TNF-α* gene exhibits an important functional polymorphism located at position −308 in its promoter region. This genetic variation results in two allelic forms, in which the presence of guanine (G) defines the common variant, but adenine (A) defines the less

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common variant. Single nucleotide polymorphisms of TNF-α are found in regions that regulate transcription or post-transcriptional events and seem to be functionally significant.[21–23] Analysis of TNF-α polymorphisms have shown evidence of this cytokine as a susceptibility and prognosis factor in some types of human cancers.[24–27] The −308 TNF-α promoter region polymorphism has exhibited an association with the susceptibility to head and neck carcinoma.[28–31] However, the impact of this polymorphism with the clinicopathological factors and prognosis for HNSCC has not been studied yet.

The aim of the present study was to investigate the association between −308 TNF-α promoter region polymorphisms in a sample of HNSCC, in accordance with clinicopathological factors. In addition, we also analysed the impact of all these variables on the overall survival of the individuals.

Material and methods

Ethical approval for this study was obtained from the relevant local ethics committees (Unimontes/CEP-1114/2008).

Tissue specimens and patients

This retrospective study was performed on archived tissue blocks from surgically resected HNSCC primary specimens ($n = 89$). Socio-demographical, clinicopathological, overall survival data, and samples were obtained from the Health Centre for Oncology at Montes Claros city, Minas Gerais, Brazil. All diagnoses of malignant disease were confirmed by histopathological analysis.

Anatomical site and clinicopathological staging

All HNSCC patients were classified according to the IUAC-TNM Classification of Malignant Tumours on the basis of the primary site, as described in the ICD for oncology.[32] The anatomical sites reviewed in this study included (1) oral cavity and perioral region (all: 40 patients/44.9% – floor of the mouth: 10/25%, tongue: 15/37.5%, retromolar trigonum: 5/12.5%, lip: hard/soft palate: 4/10%, gum: 2/5%, lower lip mucosa: 4/10%), (2) oropharynx ($n = 25$/28.2%), (3) hypopharynx ($n = 6$/6.7%), and larynx ($n = 18$/20.2%). The lesions located in the oral cavity were considered as the anterior group and those located in the oropharynx–hypopharynx–larynx as the posterior group for characterisation of the anatomical site variable. According to the TNM staging, HNSCC patients were staged as I ($n = 3$/3.4%), II ($n = 22$/24.7%), III ($n = 31$/34.8%), and IV ($n = 33$/37.1%). Morphological grading of the HNSCC group was on the basis of the WHO criteria.[33]

Evaluation of the performance status

The Eastern Cooperative Oncology Group-Performance Status (ECOG-PS) scale was used to evaluate the functional status of the HNSCC patients. The ECOG-PS scale is used to measure physical functioning as well as medical care requirements in patients with cancer.[34,35] The ECOG-PS scale categorises cancer patients into five groups: 0, normal activity; 1, strenuous activity restricted; 2, up and about for >50% of the waking hours; 3, confined to bed/chair for >50% of the waking hours; 4, 100% bedridden; and 5, dead. This validated and reliable instrument has offered additional support as a prognostic indicator.[36,37] This evaluation was rated by only one head and neck surgeon at the time of diagnosis. HNSCC samples were divided into two groups according to the ECOG-PS evaluation (0–1: good and 2–4: poor).

Genotyping for −308 TNF-α promoter region single nucleotide polymorphisms

DNA was isolated from 10 tissue sections (thickness: 10-μm) from each tissue block of the HNSCC specimens by using the DNeasy Tissue Kit (Qiagen, Chatsworth, California, USA) in accordance with the manufacturer's protocol. The allelic and genotypic polymorphisms of the −308 TNF-α promoter region were evaluated according to previously described methods.[38] Briefly, polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) used a forward primer (5′-TCCTCCCTGCTCCGATTCCG-3′) and a reverse primer (5′-AGGCAATAGGTTTTGAGGGCCAT-3′). The PCR mixture consisted of about 0.2 μg of genomic DNA, 0.2 mM dNTPs, 0.3 μM of each primer, 1.5 mM $MgCl_2$, 1×·Taq polymerase buffer, and 0.5 U of Taq DNA polymerase (PhoneutraBiotecnologia®, BH, Brazil); the final volume was 50 μL. The PCR–RFLP products were separated from the unincorporated primers and dNTPs by using a commercial kit (PCRclean) and were then subjected to *NcoI* enzyme digestion (37 °C/12 h). The amplification conditions were as follows: 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s, and the final extension step at 72 °C for 10 min. The amplification yielded a fragment of 107 bp, which was digested overnight with 10 U of the restriction enzyme *NcoI* at 37 °C. After the complete digestion, the resulting DNA fragments were separated by using 6.5% polyacrylamide gel electrophoresis (PAGE) on a 1% agarose gel, stained with silver, and photographed. The allele G was cleaved by the enzyme into two fragments, one of 87 bp and another of 20 bp (GG). The haplotype GA is composed of three fragments: 107, 87, and 20 bp. The A allele was not cleaved by the enzyme and therefore, generated one fragment of 107 bp (AA) (Fig. 1).

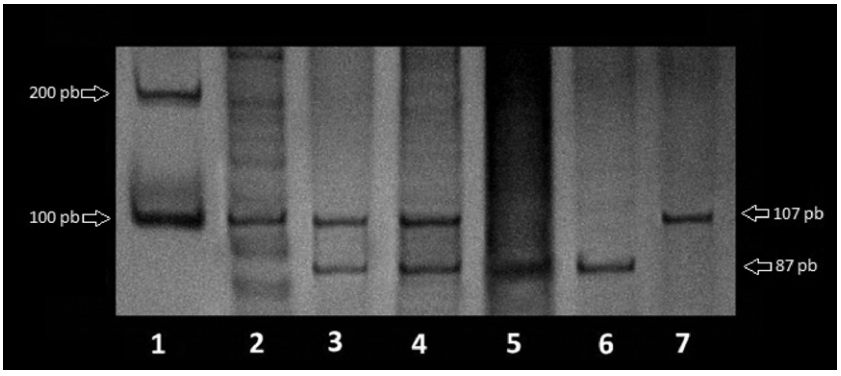

Figure 1 Polyacrylamide gel electrophoresis for detection of (308 G/A) TNF-α polymorphism. Base pair ladder (100 bp) (lane 1); PCR product without digestion = 107 bp (lane 2); genotype AA = 107 bp (lane 7); genotype G/A = 107 + 87 bp (lanes 3 and 4); genotype GG = 87 bp (lanes 5 and 6).

Statistical analysis

The Hardy–Weinberg equilibrium was assessed by using a goodness-of-fit chi-square (χ^2)-test for the biallelic biomarker. The following variables were included as covariates: ECOG-PS (0–1 vs. 2–4 groups), anatomical site (anterior vs. posterior), tumour size (small lesions-T1/T2 vs. large lesions-T3/T4), cervical metastasis (absent: N0 vs. present: N1/N2/N3), and the WHO morphological grading (I–II vs. III grades). In order to evaluate the association between the covariates and the molecular biomarker (TNF-α-dependent variable), bivariate analyses were performed by using the Mantel–Haenszel Pearson χ^2 test.

Concerning to overall survival analysis, all deaths occurred by HNSCC. Patients who died without evidence of recurrence/metastasis were considered censored by the last clinical evaluation. Five-year survival rates were estimated and survival curves were plotted according to the Kaplan–Meier test. Differences were tested by the log rank test. Multivariate survival analyses were accomplished using Cox's proportional hazards regression model to access independent prognostic factors. Only covariates with $p < 0.02$ were considered one at a time for entry into the multivariate models by using a backward step-wise procedure. The final model represented the most restricted subset of variables having prognostic significance. Associations were expressed as odds ratio (OR) values with the corresponding 95% confidence interval (CI95%). Statistical significance was set at $p < 0.05$. All statistical analyses were performed by using the SPSS 13.0 package (SPSS Inc., Chicago, USA).

Results

The socio-demographical, clinicopathological, and molecular characteristics of the HNSCC sample

Distribution of the socio-demographical, clinicopathological, survival data, and alleles and haplotypes of −308 TNF-α is exhibited in Table 1. The male-to-female ratio in the sample was 9:1. The mean age was 60.34 (±12.56 years), and median age was 61 years (range: from 33 to 91 years).

Table 1
Baseline characteristics of the HNSCC patients investigated in this study.

Variables	*n*	%	Variables	*n*	%
Age			*Gender*		
Young	16	18	Female	9	10.1
Older	73	82	Male	80	89.9
Tobacoo habit			*Alcohol habit*		
Present	25	28.1	Present	43	48.3
Absent	64	71.9	Absent	46	51.7
Cancer family			*Weight loss*		
Present	52	58.4	Absent	77	86.5
Absent	37	41.6	Present	12	13.5
ECOG			*Anatomical site*		
Good	65	73.0	Anterior	40	44.9
Poor	24	27.0	Posterior	49	55.1
Tumour size			*Cervical metastasis*		
T1/T2	34	38.2	Absent	44	49.4
T3/T4	55	61.8	Present	45	50.6
Overall survival			*WHO grade*		
⩽700 days	45	50.6	I	22	24.7
>700 days	44	49.4	II	38	42.7
			III	29	32.6
(−308G)TNF-α genotype			*Carrier allele A*		
GG	46	51.7	Absent (GG)	46	51.7
GA	31	34.8	Present (GA/AA)	43	48.3
AA	12	13.5			

Association of HNSCC with allelic and genotypic −308 TNF-α polymorphisms

The −308 TNF-α haplotype frequencies in this HNSCC sample loci exhibited an insignificant χ^2-value in the Hardy–Weinberg test ($\chi^2 = 3.024$, $p = 0.082$). Table 2 shows bivariate comparisons between the clinicopathological independent variables and allelic and genotypic (−308A) TNF-α polymorphisms. Our results showed that poor ECOG-PS (OR = 2.82, $p = 0.039$), lesions located on the posterior sites of the head and neck (OR = 4.02, $p = 0.002$), and large-size tumours (OR = 2.91, $p = 0.015$) exhibited a significant association with HNSCC individuals carrying 1 or 2 TNF-α A alleles (GA/AA). Notably, HNSCC individuals carrying the AA haplotype exclusively showed a significant association with poor ECOG-PS (OR = 6.667, $p = 0.007$) (Table 3).

Prognostic significance

The HNSCC patients were followed up for a time that ranged from 2 to 3835 days (mean: 982.1 ± 851.9 days and median: 669 days). At the time of the last evaluation (1826 days–5 years), it was noted that 45 patients (50.6%) were alive and 44 patients (49.4%) died because of head and neck carcinoma. A total of 25 (28.1%) patients were censored during the follow-up after a time period ranging from 42 to 1765 days (mean: 939.7 days: ±550.1). In the bivariate analysis, covariates such as worse ECOG-PS ($p = 0.04$), large-size tumour ($p = 0.000$), metastatic disease ($p = 0.000$), and GA genotype ($p = 0.041$) exhibited a significant association with the overall survival (Fig. 2). Subsequently, using Cox's regression multivariate models, significant differences were noted when we tested the relationship between covariates such as large-size tumour (OR = 4.87, $p = 0.005$) and presence of cervical metastasis (OR = 2.50, $p = 0.018$) with the overall survival. Our results did not show a significant association between the −308 TNF-α polymorphisms and overall survival (data not shown).

Discussion

A background of genetic and environmental factors modulates the biological behaviour of cancer. Among them, DNA polymorphisms in low penetrance genes and their cooperative interaction with related exogenous factors have been appointed as the pivotal modulators of the biological behaviour of malignancy.[39,40] In the

Table 2
Analysis between the socio-demographical and clinicopathologic covariates according to the allelic (−G308)TNF-α polymorphisms in the HNSCC sample.

Variables	(−308)TNF-α allelic frequencies			
	G	A	OR (CI95%)	*p* Value
ECOG-PS				
Good	38 (58.5%)	27 (41.5%)	2.82 (1.06–7.51)	**0.039***
Poor	8 (33.3%)	16 (66.7%)		
Anatomical site				
Anterior	28 (70%)	12 (30%)	4.02 (1.65–9.80)	**0.002***
Posterior	18 (36.7%)	31 (63.3%)		
Tumour size				
T1/T2	23 (67.6%)	11 (32.4%)	2.91 (1.19–7.22)	**0.015***
T3/T4	23 (48.1%)	32 (58.2%)		
Cervical metastasis				
Absent	22 (50%)	22 (50%)	0.88 (0.38–2.01)	0.459
Present	24 (53.3%)	31 (46.7%)		
WHO grade				
I–II	34 (56.7%)	26 (43.3%)	1.85 (0.76–4.55)	0.130
III	12 (41.4%)	17 (58.6%)		

* Values bearing asterisks are statistically significant using the Mantel–Haenszel Pearson χ^2 test.

Table 3
Analysis between the clinicopathological covariates according to the haplotype (−G308)TNF-α polymorphisms in the HNSCC sample.

Variables (*n*/%)	(−308GA) TNF-α genotype frequencies			
	GG	GA	AA	*p* Value
ECOG-PS				
Good	38 (58.5%)	22 (33.8%)	5 (7.7%)	0.231
Poor	8 (33.3%)	9 (37.5%)	7 (29.2%)	**0.007***
OR (CI95%)	Referent	1.942 (0.173–5.780)	6.667 (0.04–26.32)	
Anatomical site				
Anterior	28 (70%)	7 (17.5%)	5 (12.5%)	0.356
Posterior	18 (36.7%)	24 (49%)	7 (14.3%)	0.238
OR (CI95%)	Referent	5.35 (0.07–14.93)	2.18 (0.13–7.94)	
Tumour size				
T1/T2	23 (67.6%)	9 (26.5%)	2 (5.9%)	0.546
T3/T4	23 (48.1%)	22 (40%)	10 (18.2%)	0.052
OR (CI95%)	Referent	2.45 (0.16–6.41)	5.00 (0.04–25.64)	
Cervical metastasis				
Absent	22 (50%)	14 (31.8%)	8 (18.2%)	0.818
Present	24 (53.3%)	17 (37.8%)	4 (8.9%)	0.251
OR (CI95%)	Referent	1.11 (0.36–2.78)	0.46 (0.58–1.74)	
WHO grade				
I-II	34(56.7%)	17 (28.3%)	9 (15%)	0.086
III	12(51.7%)	14 (34.8%)	3 (13.5%)	0.939
OR (CI95%)	Referent	2.33 (0.89–6.14)	0.94 (0.25–4.08)	

* Values bearing asterisks are statistically significant using the Mantel–Haenszel Pearson χ^2 test.

current study, it was found that HNSCC individuals carrying 1 or 2 A alleles showed association with clinical factors of high severity such as large-size tumour, poor performance status of patients, and occurrence of carcinoma on the posterior sites of the upper aerodigestive tract.

Some *in vitro* and *in vivo* studies have shown that TNF-α might actively contribute to the tissue remodelling and stromal development necessary for tumour growth and spread.[18,19,41] When produced chronically by tumour–tumour, immune, and resident stromal cells, the TNF-α cytokine may act as an endogenous tumour growth factor by promoting cancer-cell proliferation through the activation of the nuclear factor kappa-β, PI3K/PKB (Akt), and the mitogen-activated protein kinase-dependent pathways.[42] Besides, TNF-α also promotes tumour growth by stimulating endothelial-cell differentiation and extracellular matrix production or stimulating other infiltrating cells to produce angiogenic factors.[43,44] According to our findings, it is possible that HNSCC individuals carrying the high producing A allele might present local tissue conditions on the upper aerodigestive tract mucosa that favours the growth and development of SCC.

Our findings also exhibited significant associations between both the high producing A allele and the AA haplotype with worse functional status in the HNSCC patients. Interesting *in vitro* and *in vivo* studies have shown evidence of the contribution of the TNF-α cytokine in malnutrition, weight loss, and cachexia disturbances in cancer patients.[45–50] It must be emphasised that there are limits for the characterisation of each one of these disturbances. While the evaluation of functional status performance is essentially clinical, the disturbances of weight loss and cachexia in cancer patients involve several, but still imprecise, energetic and metabolic abnormalities.[51] However, it is rather reasonable to make certain inferences about the possible participation of TNF-α in the performance status of the HNSCC sample in this study. The persistent inflammatory response of the host, in conjunction with appropriate production and release of cytokines such as TNF-α, IL-1, and IL-6 is central to weight loss and cachexia in cancer patients.[51,52] TNF-α expression might be induced by procachectic factors like proteolysis-inducing factor and activator protein-1, which are the transcription factors involved in promoting cachexia.[19,53,54] After its expression, TNF-α participates in the recruitment and degranulation of inflammatory cells that contribute actively to tissue degradation, especially acting on protein substrates, as noted frequently in the degradation of skeletal muscle tissue in cancer patients.[55–59]

Approximately 25% of the HNSCC patients worldwide are associated with high-risk human papillomaviruses (HPV)-16, 18, 31, 33, and 35.[60] Interesting, high-risk HPV-positive infection (60–70%) has been associated with SCC located in the oropharyngeal and larynx regions.[60–65] In our study, our HNSCC samples were classified as the posterior group; about 88% of the lesions were located in the oropharyngeal and larynx and exhibited a higher OR with individuals that presented the A allele and AA haplotype. It has been shown that HPV-transformed cells escape the host immune-cell control during carcinogenesis and become resistant to TNF-α mediated cytotoxicity. Additionally, the cytotoxicity promoted by TNF-α apparently stimulates the proliferation of HPV-immortalised epithelial cells.[66] We hypothesise that a possibly high expression of TNF-α, characterised by the presence of the high producer A allele, in association with the presence of HPV infection, could favour an immunosuppressive scenario to the HNSCC development in this anatomical site. However, that hypothesis needs to be confirmed in further studies.

HNSCC patients frequently exhibit a worse overall survival with about just 60% of all patients reaching the 5-year survival.[2–6] We found that HNSCC individuals with large-size tumours and with metastatic disease presented worse overall survival. These findings are consistent with fundamental concepts that establish the contribution of tumour size and lymph-node metastasis factors to the HNSCC outcome. A few studies showed that the GA haplotype is strongly associated with the susceptibility of some types of human cancer[67–69], including oral SCC.[28] Additionally, the AA/GA haplotypes of the TNF-α polymorphisms are generally associated with poor prognosis in patients with lymphoma.[70,71] Although, by using bivariate tests ($p = 0.041$), we observed that the GA haplotype exhibited association with worse overall survival in our sample, neither allelic nor −308 TNF-α haplotypes had an impact on the overall survival using multivariate statistical tests. Future studies are needed to foster a better understanding about the participation of TNF-α in the outcome of patients with HNSCC.

In conclusion, this study identified that HNSCC individuals carrying the high producer A allele and the high risk AA haplotype for the −308 *TNF-α* gene might exhibit local/systemic biological condi-

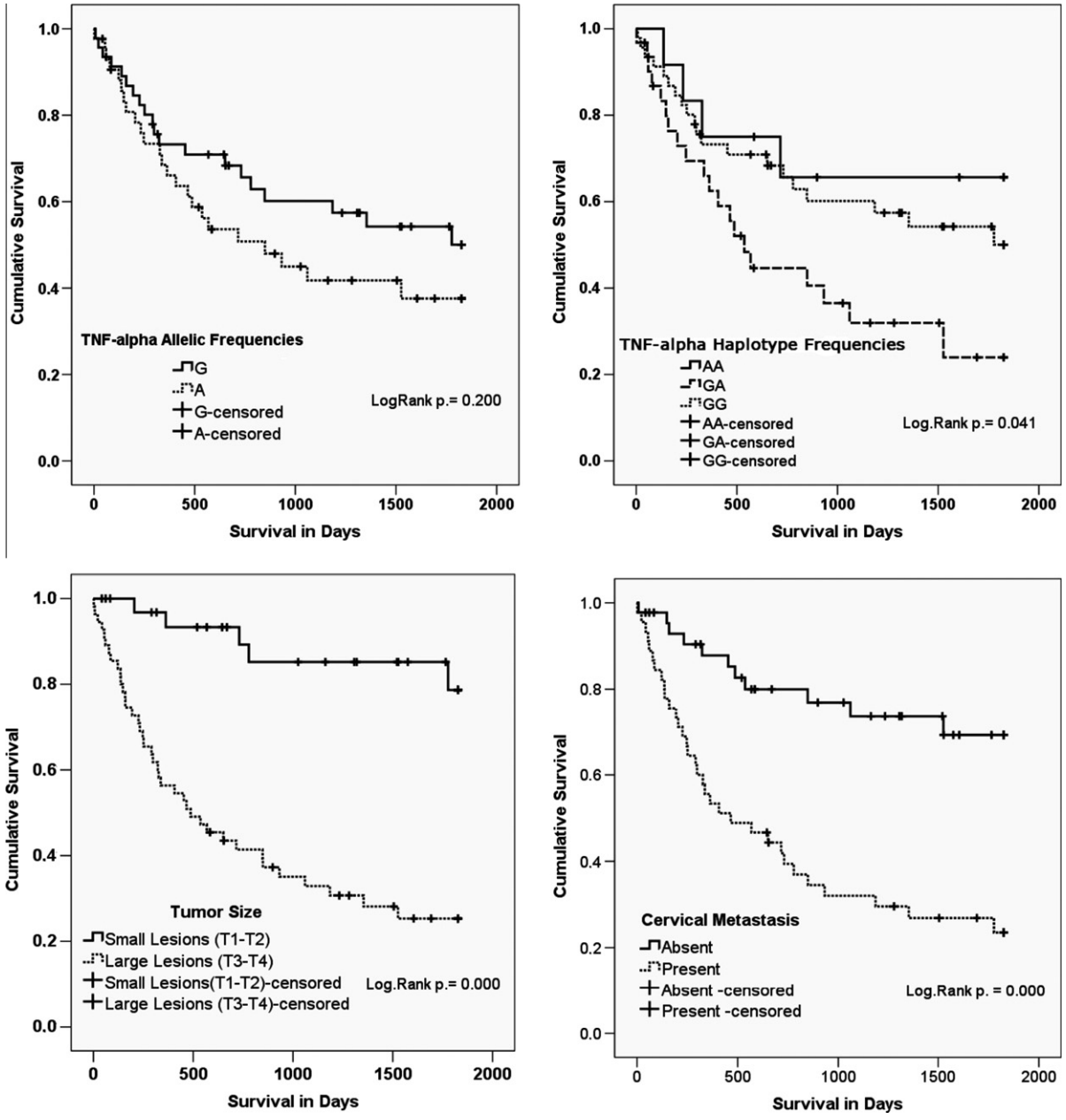

Figure 2 Overall survival analysis in HNSCC patients after 1826 days according to TNF-α allele and haplotype frequencies, tumour size, and cervical metastasis parameters. The difference between groups was evaluated by the log-rank test and the differences were statistically significant.

tions that enable the higher clinical aggressiveness of HNSCC. Poor performance status, lesions located on the posterior sites of the head and neck, and large-size tumours were associated with HNSCC individuals carrying 1 or 2 TNF-α A alleles. Neither allelic nor genotypic TNF-α polymorphisms showed impact on the overall survival of HNSCC patients in this study.

Conflict of interest statement

The authors declare no competing interests.

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